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US ARMY MEDICAL RESEARCH LABORATORY

FORT KNOX, KENTUCKY

REPORT NO. 603

THE EFFECTS OF RADIO-FREQUENCY ENERGY ON CORYNEBACTERIUM DIPHTHERIAE AND CLOSTRIDIUM WELCHII TOXINS

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UNITED STATES ARMY
MEDICAL RESEARCH AND DEVELOPMENT COMMAND

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The animals used in this study were handled in accordance with the "Principles of Laboratory Animal Care" established by the National Society for Medical Research.

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ABSTRACT

THE EFFECTS OF RADIO-FREQUENCY ENERGY ON *CORYNEBACTERIUM DIPHTHERIAE* AND *CLOSTRIDIUM WELCHII* TOXINS

OBJECT

To determine changes in toxicity and antigenicity of exotoxins after exposure to microwaves.

RESULTS

Cl. welchii, type A toxin was not affected by radio-frequency exposure, as determined by human serum opacity and mouse lethality studies. The toxicity of *C. diphtheriae* toxin was slightly reduced, while no change in antitoxin combining capacity or antigenicity was apparent. The efficacy of the available assay procedures is questioned.

CONCLUSIONS

Further studies are needed to elucidate the effects of radio-frequency on toxins. More precise and quantitative assay procedures need to be developed.

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THE EFFECTS OF RADIO-FREQUENCY ENERGY ON *CORYNEBACTERIUM DIPHTHERIAE* AND *CLOSTRIDIUM WELCHII* TOXINS

The biological effects of microwave radiation have been a source of interest and concern to workers in many fields. As early as 1891, a group of French workers reported that treatment of diphtheria toxin with shortwave radiation resulted in diminution of toxicity. Since that time, microwave radiation has been used as an experimental tool in the study of many biological and physiological systems. Interest in the effects of microwaves has been further engendered by its widespread use in industry, communications, and medicine (e.g., radar, diathermy, radio), and the consequent possible risk and hazard to personnel involved. Workers in the field have reported decreased longevity and acute lethal effects of microwaves in some mammals (1). Changes in gonadal functions of rats have been reported (2), as has inhibition of cell differentiation in developing chick embryos (3). Changes have also been produced in paper electrophoretic patterns and antigenic reactivity of human gamma globulin after exposure to radio-frequency energy (4). Changes in colloidal systems, such as starch and glycogen have been produced by microwave energy, and decrease in activity of enzymes such as amylase has been effected (5).

In 1932 a number of workers reported that ultrahigh-frequency radiation was capable of producing attenuation of three major bacterial toxins--diphtheria, tetanus, and botulinus--in raw broth filtrates. This effect occurred without the development of temperatures that might affect the potencies of toxins. These workers used skin reaction in guinea pigs to assay the toxin, and found that the minimal dose producing visible skin reaction could be increased tenfold by microwave irradiation at 158 megacycles (6). The study described herein is an attempt to confirm these observations with *C. diphtheriae* toxin, and an evaluation of the effects of microwaves on *Cl. welchii* toxin (type A).

METHODS AND MATERIALS

Toxins. Crude diphtheria toxin in broth filtrate was obtained through the courtesy of Lederle Laboratories Division, American Cyanamide Company, Pearl River, New York (Lab #42329-6063-6064). The toxin had an alleged value of 1000 guinea pig minimal lethal doses (GP MLD) per cc and 41 Lf units per cc. Clostridium toxin, dry, in sealed ampules was obtained through the courtesy of Corn States

Laboratories, Omaha, Nebraska. Diphtheria toxin for flocculation standard containing 16.5 Lf units per cc was also obtained from Lederle Laboratories.

Flocculating Serum. Standard flocculating serum labeled as containing 475 Lf units per cc was obtained through Lederle Laboratories. The serum was diluted with saline to 200 units per cc before use in the flocculation tests.

Diluents. Various diluting media were prepared in our laboratory and are further described in the descriptions of the assay procedures.

Microwave Generation. The generating system was similar to that described by Bach et al (4). The energy source was a Hewlett-Packard signal generator (608D). The energy was amplified through three low-power and one high-power wide band amplifiers, and the signal passed through two coupler and power meters. The signal was then passed through a tuning box and applied to two brass electrodes which formed the sides of the exposure chamber. This is graphically shown in Figure 1.

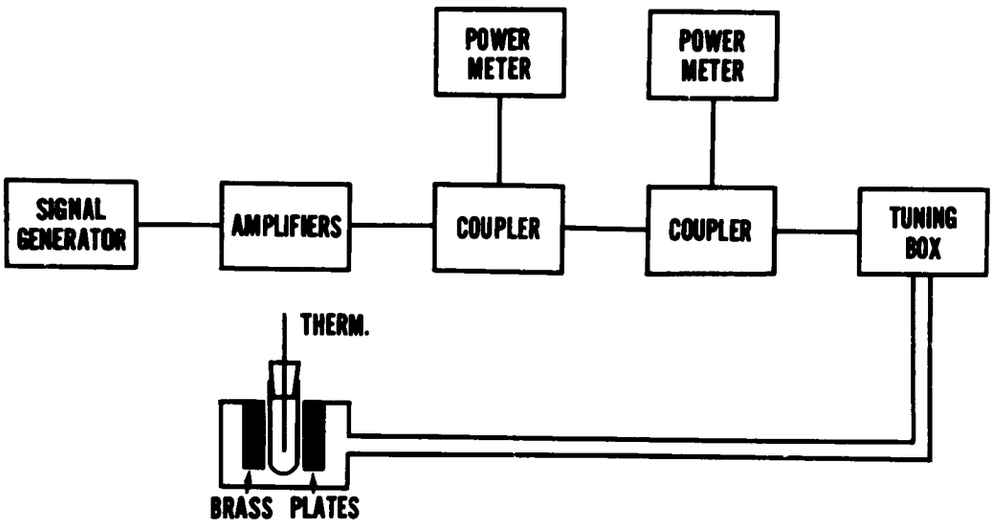

Fig. 1. Microwave generating system and exposure chamber.

Protocol of a sample exposure of diphtheria toxin is shown in Table 1.

TABLE 1

Sample Exposure Protocol, Diphtheria Toxin

Exposure	Starting Temp.	Final Temp.	Time	
I	5.0°C	37.5°C	5'30"	Toxin: 3 cc DT, crude
II	5.5°C	38.2°C	6'40"	Power: 50 watt down/ .2 watt
III	5.0°C	37.4°C	6'20"	reflected
IV	5.0°C	37.4°C	5'50"	Frequency: 158 megacycles/
V	5.2°C	37.6°C	5'20"	sec
		Total	29'30"	

Exposures. The power generated was generally 45 to 50 watts with 0.1 to 2 watts reflected. Three cc volumes of toxin were placed in 11 x 75 mm tubes. The tubes were then placed in the exposure chamber and subjected to continuous wave exposure at 158 megacycles per second. Exposure was continued for an average of 5.6 minutes until a temperature of 38°C was obtained. A control tube of toxin was heated in water bath at a similar rate to the same temperature. Both tubes were then immediately cooled to 5°C, and exposures repeated for a total of 30 to 36 minutes. (Some exposures were also made for 90 minutes and subsequently assayed for change in toxicity.)

Experimental Animals. White Swiss mice and albino guinea pigs bred in our laboratory animal division were used. White, male leghorn chicks, 1 day old, were obtained from a commercial hatchery.

PART I. DIPHTHERIA TOXIN

ASSAY OF TOXICITY BY MOUSE INTRACEREBRAL INOCULATION

It has been shown that mice inoculated intracerebrally with living cultures of toxigenic diphtheria bacilli or toxic culture filtrates showed characteristic responses which could be specifically neutralized with diphtheria antitoxin (7). According to this original description, the minimal dose that killed almost all the mice corresponded fairly well with 1 GP MLD. Kondo et al (8) further elucidated this technique and devised a quantitative method of titration based on parallel line assay. The technique consists of intracerebral injection of known amounts of toxin into mice and subsequent observation for a period of 8 days. Characteristic symptoms appear within 3 to 4 days, depending on the dose injected. We adapted this technique for our titration and found it to be relatively simple and generally replicable.

Method. White Swiss mice, 13 to 16 grams, of random sex were anesthetized until limp, with fresh ether and injected intracerebrally with .028 cc of 1:5 dilution of toxin. (Preliminary titrations with our lot of diphtheria toxin showed that injection of .028 cc of 1:5 dilution uniformly killed 70% - 95% of the mice within 8 days; this dose corresponded to approximately 4 GP MLD of toxin.) One-quarter cc syringes calibrated to .01 cc were used with a 25 gauge, 1/4 inch needle. A piece of rubber stopper was used as a shoulder on the needle, leaving 2-1/2 mm of needle exposed (Fig. 2). This was done to insure constant distance of insertion of the needle.

All injections were made in the right cranium through the median suture, when possible. The same individual made all injections in any one experiment. Experiments with injection of crystal violet showed that where the needle was inserted 2-1/2 mm, dye was subsequently found in the ventricles, whereas with a distance of less than 2 mm, the dye was at the gyri but not in the ventricles. This was in accord with studies done by Kondo et al (8).

Mice were observed for a minimum of 8 days and graded twice daily.

In a separate experiment, groups of 10 mice each were injected with saline and with heat-inactivated toxin, respectively. They showed no initial ill effects of injection and remained alive and well throughout the period of observation. Thus, the injection technique, per se, would seem to play no role in the effects subsequently observed in toxin-injected mice.

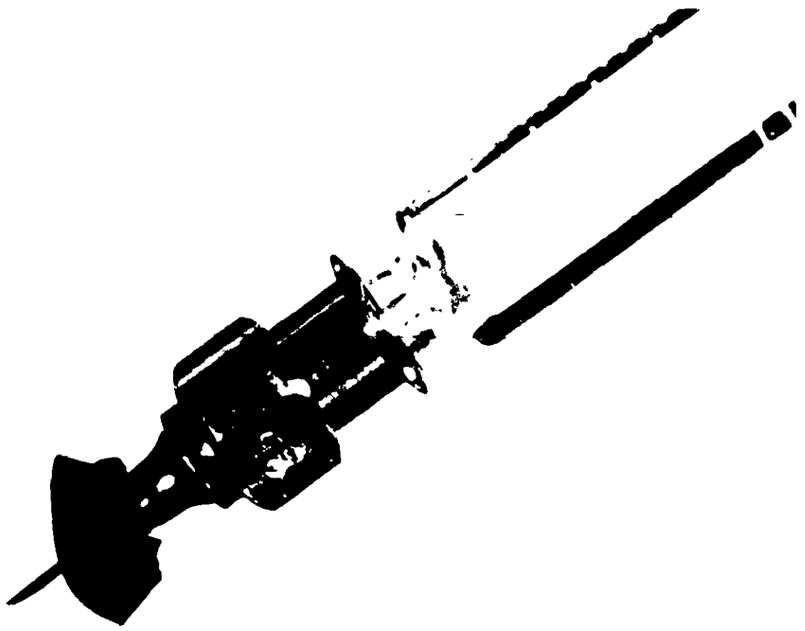

Fig. 2. Needle with rubber shoulder used for intracerebral inoculation.

Three individual experiments were performed using the sum total of 106 mice in the exposed group and 105 mice in the control groups. Responses were graded as follows:

Death.

Severe symptoms - agonal, general paralysis, or clonic convulsions.

Mild symptoms - mild paralysis or paresis, hyperirritability, or tic-like movements.

No symptoms.

Results. The results are listed in Tables 2, 3, and 4. The responses show no difference at a high level of significance, although somewhat suggestive of a decrease in toxicity with exposure.

TABLE 2

Effects of Intracerebral Inoculation of Exposed and Unexposed Diphtheria Toxin in Mice

Results		Experiment A Group Control	Exp.	B Group Control	Exp.	C Group Control	Exp.
Day of Death	5	3	4	1	1	0	0
	6	7	9	0	0	4	12
	7	13	10	11	6	15	15
	8	13	8	4	2	6	4
Survived	Severe symptoms	2	1	1	1	1	0
	Mild symptoms	4	4	2	7	8	2
	No symptoms	8	14	1	3	1	3
Total		50	50	20	20	35	36

TABLE 3

Summary of Experiments, A, B, and C

Results		Group Control A, B, C	Exposed A, B, C
Day of Death	5	4	5
	6	11	21
	7	39	31
	8	23	14
Survived	Severe symptoms	4	2
	Mild symptoms	14	13
	No symptoms	10	20
Total		105	106

TABLE 4

Death vs. Survivors in all Experiments

Results	Control	Exposed
Died	77	71
Survived	28	35
$\chi^2 = 1.05$, df = 1, $p > .15$*		

*One-tailed ($p = .15$, $\chi^2 = 1.07$).

In order to delineate any difference in histopathological effect of exposed vs. control toxin, brains were removed (9) when advanced paralysis was evident and examined microscopically. Saline-injected brains were also examined.

In the "normal," saline-injected brains, no outstanding lesions were recognized. An oblique fissure at the injection site was seen. Along the route of this fissure were cells laden with blood pigment and a minimal amount of extravasation thought to be incident to injection. In a few small areas, some pyknotic nuclei and mild gliosis were also seen.

In the toxin-injected brains, a number of lesions were evident. In general, changes were most marked in the hippocampal gyri. There was congestion of venous channels. Marked necrosis of individual cells was evident and these lesions were bilaterally symmetrical. Diffuse demyelination, satellitosis, gliosis, and some neuronophagia were also present in most sections. These changes were found in brains injected with either the control or exposed toxins and no qualitative or quantitative differences between the two were observed.

ASSAY OF DIPHTHERIA TOXIN USING CHICKS

While retesting the pathogenicity of diphtheria organisms for various species of birds and animals, it was noted that 7 day old chicks regularly succumbed within 48 hours following intraperitoneal or subcutaneous inoculation of rabbit virulent strains (10). One GP MLD of diphtheria toxin regularly killed the chicks and diphtheria antitoxin afforded significant protection. Paralysis of wings and legs was a prominent response, usually leading to death or survival with paresis. The

chick method has been applied for the titration of toxins and antitoxins and is thought to be somewhat more precise than the mouse method (8, 11).

Method. Raw toxin containing approximately 750 GP MLDs per cc was used. Exposed and control samples were diluted with phosphate buffered saline, pH 6.8 to 5 MLD per cc.

White, male leghorn chicks were obtained from a local hatchery when they were 1 day old. They were kept at constant temperature and fed standard chick food obtained commercially. At 7 days of age the chicks were paired by weight and divided into two equal groups. Injections of .75 cc of toxin were made intraperitoneally, using 1 cc syringes with 26 gauge needles. The chicks were observed for 5 days and graded as follows:

Death on first day.

Death on second day.

Death on third day.

Survival with symptoms.

Survival with no symptoms.

Results. Initial studies showed that 2-4 GP MLDs were generally lethal to more than 50% of the chicks. With doses less than 2 GP MLDs or greater than 4 GP MLDs, effects were inordinately variable with no replicable results apparent. Further preliminary studies also showed that prior injection of the chicks with antitoxin generally protected the chicks from subsequent death or paralysis.

It should be noted at this point that we did not think the chick method as precise and as replicable as suggested by some reports in the literature. It was difficult to maintain adequate control groups. At times chicks that received no inoculation whatever became ill or died (2 - 3% of groups of > 100 chicks). On a few occasions, large doses of toxin failed to produce any effect on some chicks while proving lethal within a few hours to another group. These difficulties arose despite careful attempts to maintain ideal conditions of temperature, humidity, adequate food and water, etc. The aforementioned difficulties notwithstanding, we felt that the observations and data obtained from two experiments using 225 chicks were of interest, as will be seen from the results. The effects noted in two experiments are shown in Tables 5 and 6. Fatalities

TABLE 5

Effects of Inoculation of Exposed and Unexposed Diphtheria Toxin in Chicks

Results		Experiment I Group Control	Experiment I Group Exposed	Experiment II Group Control	Experiment II Group Exposed
Day of Death	1	29	21	36	35
	2	3	1	0	0
	3	2	1	1	0
Survived	Symptoms	1	2	2	3
	No symptoms	15	25	23	23
Total		50	50	62	61

TABLE 6

Summary of Experiments I and II on Effects of Chick Inoculation with Diphtheria Toxin

Results	Control	Exposed	Total
Died	71	58	129
Survived	41	53	94
Total	112	111	223

$\chi^2 = 2.86$, df = 1, p = .05 (one-tailed test)

and survivors in control and exposed groups are identical in Experiment II. There is a larger number of survivors in the exposed group in Experiment I. Statistical analysis of both experiments combined reveals that the difference is significant, albeit at a low level of probability (p = .05). There is no apparent explanation for the discrepancy between experiments.

ASSAY OF DIPHTHERIA TOXIN USING GUINEA PIG SKIN TESTS

The response of guinea pigs to diphtheria toxin is so variable that the use of the "animal unit" in the titration of diphtheria toxin is relatively inaccurate. The minimal reacting dose (MRD) is preferable to the MLD, in that it is economical and eliminates animal variation because both standard and unknown toxins can be assayed in the same animal. Nevertheless, the end point is still arbitrary and is influenced by many nonspecific factors. It has recently been shown that a linear relationship exists between the diameter of the skin reaction elicited to the log dose of diphtheria toxin injected (12). Thus, assay of toxicity of a toxin may be performed by comparing diameters of skin lesions elicited by intradermal injections of the toxins in guinea pigs.

Methods. Albino guinea pigs weighing from 450 grams to 750 grams were shaven with electric clippers. A depilatory agent was applied and spread with a spatula over the clipped areas. This was washed away with warm water 5 minutes after application, and the skin was then dried gently with towels. The depilated areas were divided into eight spaces.

Two doses of each of the toxins (exposed and unexposed) were prepared. The high dose was 1:300 dilution and the low dose, 1:3000 dilution, phosphate buffer saline being used as diluent. (Preliminary studies showed that the low dose gave lesion diameters of at least 10 mm.) In order to eliminate site and animal variations as a source of error of estimated potency, each of the doses was injected in duplicate into every animal and at each of the eight possible sites.

Intradermal injections of .2 cc were made. The diameter of the resulting lesions was measured with a transparent ruler after 24 hours. The long and short diameters of elliptical lesions were measured and the square root of their product was reported as the diameter. A total of 10 guinea pigs were used in two separate experiments.

Results. The duplicate diameters of the skin reactions, the mean diameters, and the respective standard deviations are shown in Table 7. The means of both high and low dose control toxins are slightly greater than those of the exposed toxin. Examination of the table reveals that in animals 4, 8, and 9, there are some dubious and unexplained results. In #4, the E^L diameter is greater than E^H. In #8 and #9, both low dose diameters exceed the high dose result. It seemed reasonable, therefore, to exclude these animals and evaluate the remainder.

Table 8 shows evaluation, by sign test, of all the subjects, and of those after exclusion of animals 4, 8, and 9. In both groups, comparison

TABLE 7

Diameter of Skin Reactions, in Millimeters, following Intradermal Injections of Control and Exposed Toxins

Toxin Dose	Guinea Pigs 1	2	3	4	5	6	7	8	9	10	Mean σ
C^H	21 18	18.5 21	21 20	18.5 20	16.5 16	19 20.5	24.5 29	20 22	24 22.5	23 22.5	20.9 ± 2.89
C^L	17 17	15.5 18	16 19	18 14.5	13.5 13	15 14	20.5 21.5	25 24.5	21.5 27	20 17	18.4 ± 3.87
E^H	19.5 21	18 19.5	16.5 20.5	16 14.5	16.5 17.5	15.5 15	25 23	24.5 20.5	27.5 22.5	22 23	19.9 ± 3.57
E^L	15.5 16.5	14 15	15 17	17 20.5	12 12	14 14	19 21	20 26	21.5 27.5	19 17.5	17.7 ± 4.08

Key: C^H - Control High Dose; C^L - Control Low Dose; E^H - Exposed High Dose; E^L - Exposed Low Dose.

TABLE 8

Evaluation of Mean Size of Lesions by Sign Test

Comparison	Dose	Number of Comparisons C > E	E > C	p
All subjects	H	6	4	.377
	L	8	2	.055
Excluding questionable subjects (4, 8, 9)	H	5	2	.227
	L	7	0	.008

Key: C - Control; E - Exposed; H - High Dose; L - Low Dose.

at the low dose level reveals a more significant difference than at high doses. These findings suggest that there may have been a slight decrease in toxicity effected by R-F exposure and that the skin test lacks sufficient sensitivity to detect such small differences at high dose levels. However, this effect was nothing like a tenfold decrease in toxicity, as reported by the earlier workers (6).

FLOCCULATION TEST

The aforementioned assay procedures are in vivo tests which determine the lethality and skin toxicity of toxins. The antigenic value of a toxin or the combining capacity of the toxin with antitoxin may be determined by the in vitro flocculation technique (13). The unit of flocculation is the Lf dose which is defined as the amount of toxin which flocculates most rapidly with 1 unit of antitoxin in a series of mixtures containing constant amounts of toxin and varying amounts of antitoxin (14).

Method and Results. The Lf value of exposed and controlled toxin was determined by well-established flocculation techniques (15). Eight separate flocculation procedures were performed, giving essentially the same results. The protocol of one flocculation test is given in Table 9. To a series of eight test tubes (5 x 50 mm) were added small amounts of antitoxin differing by .005 cc from tube to tube; .2 cc of the toxin was then added to each tube. The tubes were inverted once and placed in a water bath at 46°C. They were observed every 15 minutes for the first appearance of flocculation. The level of water in the bath was approximately one-third the height of the column inside the tube. The point of initial flocculation was designated as FF. Any subsequent flocculates were recorded as F.

Calculation of the flocculating value of the toxin from the results as shown in the protocol is as follows:

Two-tenths cc of the toxin flocculated with .035 cc of the antitoxin. Since the antitoxin contained 200 units per cc, 1 Lf unit is contained in .005 cc of antitoxin. Since the Lf is the amount of toxin that will flocculate with 1 unit of antitoxin, therefore, $\frac{.2}{.035} = \frac{X}{.005}$

X = Lf = .028 cc of 35 flocculating units per cc in both the exposed and control toxin.

A number of flocculation tests were also performed using 2 cc of toxin in each tube with proportional amounts of antitoxin (in 11x75

TABLE 9

Comparison of Antitoxin Combining Capacity of Exposed and Unexposed Diphtheria Toxin, by Flocculation Test

	TOXIN															
	Control								Exposed							
Tube No.	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
Antiserum (cc)	.025	.030	.035	.040	.045	.050	.055	.060	.025	.030	.035	.040	.045	.050	.055	.060
Toxin (cc)	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2
Time																
15, 30, 45, 60, 75 min	--	C	C	C	C	C	C	C	--	C	C	C	C	C	C	C
120 "	--	C	P	P	C	C	C	C	--	C	P	C	C	C	C	C
160 "	--	F	FF	F	C	C	C	C	--	F	FF	F	C	C	C	C
220 "	--	F	FF	F	C	C	C	C	--	F	FF	F	C	C	C	C

Key: C - Cloudy; P - Precipitate, FF - Earliest Flocculation; F - Secondary Flocculation.
Antiserum - Flocculation serum #16d6 (Lederle), 475 Lf units/cc diluted tc 200 u/cc.
Toxins - Lot #Mix. X, 16.5 Lf u/cc (Lederle).
H_2O Bath - 46°C; H_2O level one-third height of column in tubes.

mm tubes). The results were the same as with the previously described procedure. In a few of the flocculation tests the Kf or flocculation times varied somewhat but the "indicator tubes" were the same. Such variations in flocculation time have been shown to be due to difference in temperature of the water bath, pH, etc.

Attempts were also made to titrate the toxin, using micro tubes and much smaller quantities of toxin and antitoxin. However, this technique gave poorly readable end points and was abandoned.

Thus, no change in antigenicity or antitoxin combining capacity could be demonstrated in the exposed toxin, by the in vitro flocculation method.

COMPARISON OF EXPOSED AND CONTROL TOXIN BY IMMUNODIFFUSION

Immunodiffusion was carried out in order to determine whether any gross qualitative change in antigenic character of the toxin was effected with radio-frequency.

Method and Results. Ouchterlony plates were prepared as follows:

Standard petri dishes were filled with 15 cc of 1% nutrient agar. One cc wells were made in the agar, using a number 5 cork borer. A center well and four surrounding wells .6 cm from the center well were prepared. Standard flocculating antitoxin was placed in the center well while two outer wells were filled with control toxin and two with exposed toxin. The dishes were then incubated at room temperature and observed every 12 hours. Maximum precipitation bands were observed at 48 hours. These were examined with a dissecting microscope. Two thick precipitation bands appeared in front of each outer well. No differences between the control and exposed bands were noted.

PART II. THE EFFECT OF RADIO-FREQUENCY ON THE ALPHA TOXIN OF CLOSTRIDIUM WELCHII

Gas gangrene secondary to Clostridium welchii infection is not common in the general population. However, in war time, the incidence of gas gangrene in wounded servicemen has been relatively high, especially when treatment had been necessarily delayed (17).

Since the anerobes grow rapidly, death may ensue within a few hours of injury. The therapeutic value of specific gas gangrene antitoxin is not definitely proven in humans.

Attempts to produce effective toxoids for active immunization have been made and successful immunization in humans has been reported (18, 19). The chief limiting factor in successful immunization has been the production of good toxoids, difficulties arising in the concentration of potent toxins and subsequent conversion to toxoids without loss of antigenic efficiency (20, 21).

If R-F energy could be shown to effect toxicity of Cl. welchii toxin without a decrease in antigenicity, another perhaps superior method of toxoid production might be made available. Thus, the effect of R-F energy at 158 megacycles on Cl. welchii toxin was investigated.

METHODS AND MATERIALS

Toxin. Clostridium perfingens type A toxin packed in hermetically sealed glass ampules was obtained from Corn States Laboratories of Omaha, Nebraska. The alleged I. V. lethal dose in mice was approximately .2 mg (Lot II BP 6K).

Diluting Fluids. Tryptone medium (5 gm tryptone, 1.25 gm NaCl, in 500 cc distilled H_2O) was used as the diluent for the dry toxin; Borate buffered saline at pH 6.8 was used for further dilution in some of the serum turbidity tests.

Test Animals. White Swiss mice of random sex, ranging in weight from 14 to 18 grams were used.

Serum. Pooled, fat-free sera from fasting human subjects were obtained from the Ireland Army Hospital Clinical Laboratory. The serum was inactivated at 56°C for 1 hour and stored at -53°C in small aliquots until ready for use.

Exposure. Dry Cl. welchii type A toxin was carefully weighed, placed in 11 x 75 mm tubes, and dissolved in 3 cc tryptone medium. These tubes were then placed in exposure chamber as described in Part I, and subjected to R-F energy at 158 megacycles per second. Exposure was continued until a temperature of 38°C was obtained. Tubes were immediately placed in freezer until the temperature was reduced to 5°C and then exposure repeated for a total duration of 90 to

94 minutes. Numerous exposures with varying concentrations of toxin, showed that the duration of exposure necessary to attain temperature of 38°C varied directly with concentration. It was also observed that when buffered saline was used as diluting medium, exposure time to reach 38°C increased by 2 to 3 minutes for different concentrations of toxin.

Most exposures were subsequently performed with 10 mg of toxin dissolved in 3 cc tryptone medium, with individual exposures lasting 5 - 7 minutes. Unexposed control tubes were heated in water bath to 38°C and then rapidly cooled to 5°C together with the exposed tubes. Subsequent heating and cooling was repeated for the same duration as that of the exposed tubes. The exposed and control toxins were then diluted with tryptone medium or Borate buffered saline for use in the I. V. lethality tests and serum turbidity tests. Tryptone medium maintained the stability of the dissolved toxin for 2 to 3 weeks while buffered saline maintained the stability of the toxin for approximately 1 week.

INTRAVENOUS MOUSE LETHALITY TEST

A number of *in vivo* and *in vitro* methods for the titration of *Cl. perfingens* toxin are available. The intravenous injection of a standard dose of Clostridium toxin or of a toxin-antitoxin mixture containing an excess of toxin will generally result in the death of the animal (mouse) within 72 hours, the time of death varying somewhat with the dose of toxin used. The titration of toxins and of specific antitoxins may be carried out using this procedure (22).

Method. White Swiss mice weighing between 15 - 19 grams were paired by weight and placed in battery jars for 1/2 hour before injections. Exposed and control toxins were diluted with tryptone so that each cc contained .3 mg of toxin by dry weight. A volume of .5 cc of the diluted toxin (.15 mg of dry toxin) was injected into the tail vein of the mice. All injections were made by the same individual in any one experiment. Injections were made using 1 cc tuberculin syringes with 27 gauge, half-inch needles. The mice were then observed for a period of 72 hours.

An additional group of 10 mice was injected with .5 cc of tryptone and another 10 mice were injected with .5 cc of unexposed diluted toxin (.15 mg) that had been heated to 90°C in a water bath for 90 minutes. No deaths and no ill effects were observed in any of the latter two groups of mice.

Results. The observed effects in two separate experiments, using 80 and 60 mice, respectively, are listed in Table 10.

TABLE 10

Effects of Intravenous Injection of Exposed and Unexposed (Control) Clostridium welchii Toxins in Mice

Experiment	Effect	Group	
		Control	Exposed
I	Early deaths*	21	22
	Late deaths**	6	7
	Survived	13	11
II	Early deaths*	26	25
	Late deaths**	1	1
	Survived	2	0

*1. 5 - 8 hours post-injection.

**8 - 24 hours post-injection.

There are no apparent differences in death rates or survival rates between exposed and control groups of animals in either of the two experiments. In the second experiment, the death rates of both groups are significantly increased over that in Experiment I. Review of our data revealed that the mice in Experiment II were 2 - 3 grams lighter than the mice used in Experiment I. This may explain the higher death rate.

A bloody discharge was noted over the urethral meatus of nearly all of the mice that died. Necropsy of four mice in the control and exposed group that died revealed the following:

The kidneys were all grossly severely hemorrhagic and blood was found in the ureters and bladders of a number of the mice. Microscopic examination of the kidneys revealed marked necrosis of the tubular epithelium with blood and hemoglobin casts in the lumens of the distal convoluted tubules. Proximal convoluted tubules were distended with a faintly granular eosinophilic material suggestive of blood pigment. The glomeruli showed swollen endothelial cells and some

proteinaceous material in Bowman's space. The ureters, bladder, urethra, and adrenals showed no significant changes. The entire picture was compatible with an acute tubular necrosis or hemoglobinuric nephrosis seen with burns, crush injuries, or severe toxemia. There were no apparent pathological differences between the animals receiving exposed or control toxin. Necropsy was not performed on animals that survived and showed no ill-effects.

HUMAN SERUM OPACITY REACTION

While the titration of diphtheria and tetanus toxins and toxoids can be carried out with fair accuracy by means of the flocculation test, this latter has not been successfully applied with Cl. welchii toxin or toxoids.

Consistent and reproducible results in the titration of MLD doses of Clostridium toxins or toxoids by means of the human serum opacity tests have been obtained. This reaction was introduced by Nagler in 1939 (23), and further applied to the titration of antitoxin and toxoids by Seal and Stewart (24).

When graded amounts of Cl. welchii type A toxin are added to inactivated fat-free human serum, an opacification occurs after 16 hours incubation, and the degree of turbidity correlates with the amount of toxin used. This reaction is caused by the lethal toxin of Cl. welchii and cannot be produced by any other clostridial toxins. The reaction is specifically inhibited by Cl. welchii antitoxin and not by other bacterial antitoxins. (Another organism, a gram positive rod resembling pseudoanthrax bacillus has been found to change the serum in a similar manner, but the change could not be inhibited by Cl. welchii antitoxin.)

Of a number of mammalian sera tested, only human serum was found to exhibit the opacity reaction.

Method and Results. Ten mg of Cl. welchii toxin was dissolved in 2.5 cc of tryptone and exposed to R-F energy. Control toxin was treated as described above. After exposures, the toxins were diluted to .5 mg per cc (toxin A), .25 mg per cc (toxin B), and .1 mg per cc (toxin C). Dilutions were made with both tryptone and with Borate buffered saline. Numerous trials showed that the opacity reaction was more distinct and occurred at a lower toxin concentration when tryptone was used as the diluent. A series of 6 x 50 mm tubes were set in a rack and .2 cc of human serum was added to each tube. The serum was previously centrifuged at 8000 RPM for 25 minutes and any fat present was removed. Buffered saline or tryptone in increasing

increments of .01 cc was then added to the tubes, beginning with .02 cc in tube 1 and .1 cc in tube 9. Exposed and control toxin was then added to the tubes in decreasing amounts as shown in Table 11. The tubes were then stirred on a Vortex mixer for 3 seconds, replaced in the rack, and incubated at 37°C for 16 hours. Opacity was graded as +1 - +3. The results are shown in Table 11. It can be seen that there were no observable differences between the exposed and control toxins in any of the concentrations examined.

TABLE 11

In vitro Titration of Exposed and Unexposed Toxins, at Varying Concentrations, with Human Serum

Tube No.	1	2	3	4	5	6	7	8	9
Serum (cc)	.2	.2	.2	.2	.2	.2	.2	.2	.2
"Saline"* (cc)	.02	.03	.04	.05	.06	.07	.08	.09	.1
Toxin (cc)	.08	.07	.06	.05	.04	.03	.02	.01	--
Readings at 16 hours (after incubation at 37°C)									
Toxin A (.5 mg/cc) Exp	+++	+++	+++	+++	+++	+++	++	±	--
Toxin A (.5 mg/cc) C	+++	+++	+++	+++	+++	+++	++	±	--
Toxin B (.25 mg/cc) Exp	+++	+++	+++	+++	++	+	±	--	--
Toxin B (.25 mg/cc) C	+++	+++	+++	+++	++	+	±	--	--
Toxin C (.1 mg/cc) Exp	++	++	+	±	--	--	--	--	--
Toxin C (.1 mg/cc) C	++	++	+	±	--	--	--	--	--

*Tryptone used as diluent in most of the tests.

Key: Exp - Exposed Toxin; C - Control Toxin.

COMMENT

Our studies failed to reveal any effect of microwave radiation on Clostridium toxin under the conditions described. Similarly, we have

been unable to duplicate the marked effects observed by earlier workers on diphtheria toxin, although some of the assays suggest a slight decrease in toxicity after R-F exposure. It is difficult to pinpoint the reasons for the discrepancy between our results and that of earlier workers. However, some factors that may enter are rather obvious. Measurements of the effects involve at least two distinct instrumentation areas, the biological and electrical (16). Certainly the equipment used by the earlier workers differed from that used by us in the present study. Environmental factors may affect microwave generation. Nearby antennas and non-dielectric containers may interfere with energy output of a generating system. Water has been shown to interfere with energy transfer and absorption (16). Differences in concentration may affect the responses of biological systems to radio-frequency energy (4).

Of interest are recent observations that changes in magnetic field strength may alter responses to radio-frequency (25). Variations in different lots of toxins (pH, ionic strength, presence of other proteins in media) may affect responses to microwave irradiation. Similarly, the role of small variations in exposure time, frequency, and temperature has yet to be delineated.

The biological assay techniques used by the earlier workers may be subject to some criticism. The end point of the minimal reaction dose is completely subjective and arbitrary, and is influenced by many nonspecific factors, as mentioned earlier. Although our method of assay, using the skin reaction, is perhaps somewhat more objective, the wide range of responses in any one guinea pig with the same doses of toxin show that the method is not precise enough to determine small changes in toxicity. Nevertheless, if we assume that our exposure techniques were similar, we would have expected more significant differences in skin reaction, if anything like a tenfold change in skin toxicity were effected.

Contrary to suggestions in the literature (8), we felt that chicks were far from ideal for biologic assay of toxins. The responses of the chicks were far too variable to give precise dose effect curves.

We may conclude that under the conditions described and with the aforementioned variables considered, no definite effect of microwave radiation on Cl. welchii toxin was produced. Some of the assays suggest a small decrease in toxicity of C. diphtheriae toxin.

Further studies are needed to elucidate and define the effect, if any. These may have to await the development of more refined and quantitative assay procedures for toxins, and clarification of the role of variables such as magnetic field strength.

SUMMARY

Commercially obtained C. diphtheriae and Cl. welchii toxins were exposed to continuous microwave irradiation at 158 megacycles per second.

Potencies of exposed and unexposed control toxins were compared by the following assay techniques: Diphtheria toxins - intracerebral mouse inoculation; guinea pig skin reaction; intraperitoneal chick inoculation; standard flocculation studies; and immunodiffusion.

Clostridium welchii toxin - serum turbidity test, and standard mouse lethality test.

No effect on Cl. welchii toxin was noted. Some of the assays suggest a small decrease in toxicity of C. diphtheriae toxin. Further studies with more refined assay techniques may be in order to elucidate the effects.

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Captain Gilberto S. Treviño, VC, USA, examined the microscopical slides and described the pathology.

1st Lieutenant G. Korteling, MSC, USA, and Sp 4 K. Johnson performed the microwave exposures.

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